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RESEARCH ARTICLE

A natural bacterial strain *Bacillus pumilus* 16: Identification and antibiotic resistance evaluation

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Abstract

Microbial biopreparations are actively used to prevent, diagnose, and treat infectious, allergic, tumor, and autoimmune diseases in humans and animals; to stimulate the growth and development of plant crops. Natural bacterial strains with valuable technical properties are a vital biological resource for developing new biopreparations and rotating already known microbial preparations in the world market. This study describes a new natural strain *B. pumilus* 16, which was isolated from the rhizosphere of Cichorium. The strain was identified using morphological and physiological parameters, biochemical tests, and primers Pum-f. and Pum-r. Antibiotic sensitivity and antagonistic activity against *Escherichia coli* were determined by diffusion of discs and delayed antagonism methods, respectively. The new natural strain (like type strains) fermented arabinose, cellobiose, mannitol, mannose, salicin, sucrose, and trehalose, and gave a positive reaction to arginine dihydrolase, ONPG, Voges-Proskauer test. It also gave a negative reaction to inositol, raffinose, sorbitol, methyl-D-glucoside, inulin, and lecithinase. *B. pumilus* 16, unlike the test strains, was capable of fermenting citrate. Strain *B. pumilus* 16 was highly sensitive to cephalexin (37.9±0.7 mm) and enrofloxacin (25.7±8.9 mm); sensitive to oleandomycin (17.1±1.9 mm), benzylpenicillin (18.5±1.2 mm), and monomycin (16.0±0.6 mm); resistant to oxacillin. By the agar blocks method (7.3±1.5 mm), a more pronounced antagonism of the new strain against *E. coli* was recorded than by the method of agar wells (5.3±0.6 mm). Due to the level of antagonistic activity, *B. pumilus* 16 was more effective than the type strains (two of which did not show an antagonistic effect). On the basis of this, the new strain can be recommended for inclusion in the bacterial preparation composition for the national economy.

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Keywords
Bacillus pumilus, rhizosphere, antagonism, antibiotic resistance, *Escherichia coli*

Introduction

Biological preparations, including microbial bio preparations, are actively used to prevent, diagnose, and treat an infectious, allergic, tumor, and autoimmune diseases in humans and animals. Biological preparations stimulate the growth and development of plant crops (Kyrychenko 2015; Johnston et al. 2018). Natural bacterial strains with valuable technical properties are a vital biological resource for developing new biopreparations and rotation of already known microbial preparations in the world market. Currently, bacteria of the genus *Bacillus* are one of the most studied groups of microorganisms. They are widely used in various sectors of the economy (Ruiz-Garcıa et al. 2005; Baruzzi et al. 2011; Cihan et al. 2012). The genus *Bacillus* belongs to the Firmicutes phylum and includes more than 200 species. Representatives of this taxon are Gram-positive rods, capable of forming endospores (Ten et al. 2006; Zeigler and Perkins 2008). In relation to oxygen bacilli, they can be obligate aerobes and facultative anaerobes. Therefore, they are widespread (Earl et al. 2008; Connor et al. 2010; Mathew and Krishnamurthy 2018).

B. subtilis is a type of the genus *Bacillus*. It is actively used in medicine, veterinary medicine, crop production, etc. (Shahcheraghi et al. 2015; Vogt et al. 2018; Wang et al. 2018). Taxonomists even distinguish the *Bacillus subtilis* group, which, in addition to *B. subtilis*, includes the following species: *B. amyloliquefaciens*, *B. atrophaeus*, *B. axarquiensis*, *B. licheniformis*, *B. malacitensis*, *B. mojavensis*, *B. pumilus*, *B. sonorensis*, *B. tequilensis*, *B. vallismortis* and *B. velezensis* (Jeyaram et al. 2011; Alina et al. 2015). Such species as *B. licheniformis* established themselves as an effective producer of enzymes and an active antagonist against pathogenic microorganisms (Schallmey et al. 2004; Alvarez-Ordóñez et al. 2014). However, the biological activity of *B. pumilus* has not yet been adequately studied.

It is known that *B. pumilus* is highly resistant to extreme environmental conditions such as low or no nutrient availability, desiccation, irradiation, and H2O2 and chemical disinfectants. Many strains of *B. pumilus* are sensitive to tetracycline, kanamycin, erythromycin, vancomycin, and resistant to penicillin. Recently, it was also established that *B. pumilus* is marked with antifungal and antibacterial activity (Parvathi et al. 2009; Gao et al. 2017; Chu et al. 2019; Morita et al. 2019) against plant and animal pathogens. The constant search and study of active natural strains of microorganisms that are antagonists of pathogenic microbes are relevant because collections of bacterial cultures play a crucial role in mobilizing biological resources and make it possible to form a solid base for genetic, molecular, biological, and biotechnological studies.

This research aimed to identify and study the characteristics of a new natural strain isolated as an antagonist against *Escherichia coli*.

Material and methods

The bacterial type strains, namely, *B. pumilus* B-7886, *B. pumilus* B-7917, *B. pumilus* B-7919, and *B. subtilis* B-1323 were procured from the Russian National Collection of Industrial Microorganisms (VKPM). The L-broth was used for the accumulation of biomass of bacteria of the genus *Bacillus* and *E. coli* and to conduct experiments on the study of direct antagonism. L-broth is made up of yeast extract (5 g/L), peptone (15 g/L), and NaCl (5 g/L) (FBIS SRCAMB, Russia, 2018). Solid L-medium obtained by adding agar (15 g /L) to the L-broth was used to assess the purity of bacterial cultures and to study delayed antagonism and antibiotic resistance. The pH index was adjusted at 7.0±0.2.

Cultures of *Bacillus* spp. in L-broth were grown at 37 °C in shaker-incubator "Innova 44" (New Brunswik, USA, 2015) at 250 rpm for 18–24 hours. *E. coli* cultures in liquid and agarized medium and plates to study delayed antagonism and antibiotic resistance were grown on a thermostat 'Binder BD 115' (Binder, German, 2014) at 37 ° C for 18–24 hours. As research materials, more than 100 samples of the rhizosphere of plants were selected from six administrative-territorial divisions of the Altai Krai (Russia). *Bacillus* sp. strains were isolated from the rhizosphere using standard sampling methods, ten-fold serial dilutions, and the spread plate method (Eremina and Krieger 2005; Shirokikh and Merzaev 2007). The general experimental scheme is shown below (Fig. 1).

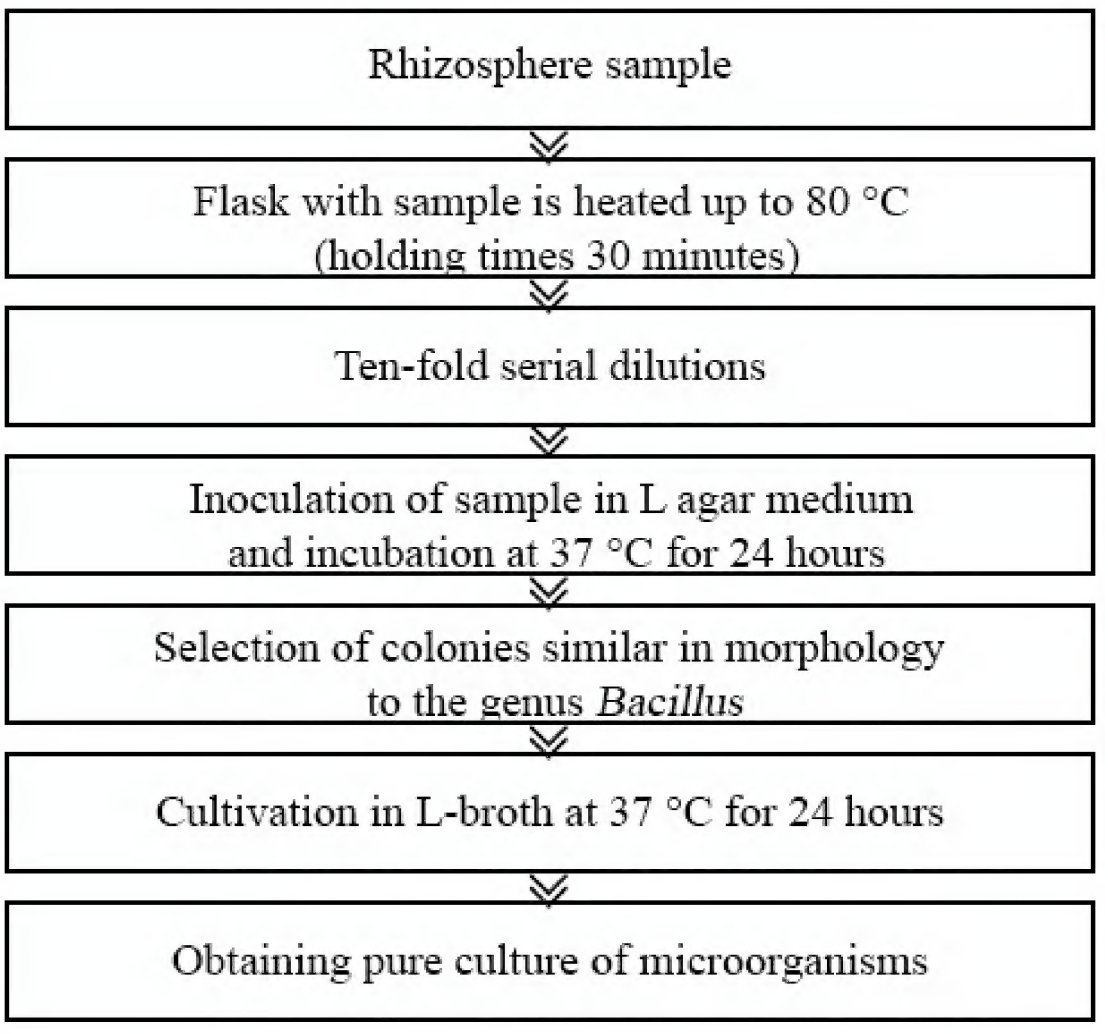
Rhizosphere sample
Flask with sample is heated up to 80 °C
(holding times 30 minutes)
Ten-fold serial dilutions
Inoculation of sample in L agar medium
and incubation at 37 °C for 24 hours
Selection of colonies similar in morphology
to the genus Bacillus
Cultivation in L-broth at 37 °C for 24 hours
Obtaining pure culture of microorganisms

Figure 1. The isolation and identification scheme of rhizosphere microorganisms.

The identification of rhizobacterial strains up to the genus *Bacillus* was carried out following the Bergey's Manual. The reaction to lecithinase established the safety of the isolated strains. It is negative for nonpathogenic representatives of the genus *Bacillus*.

Analysis of the nucleotide sequence of the 16S rRNA gene was used to confirm the belonging of the strain to the genus *Bacillus*. Subsequent screening using the GenBank and RDP-II databases was used for further identification of the species. Primers Pum-f, Pum-r, Saf-f, and Saf-r were used in PCR.

The Microgen Bacillus ID system was used for the biochemical characterization and identification of a novel strain.

The following antibiotics were used to study antibiotic resistance: cephalexin, oleandomycin, enrofloxacin, benzylpenicillin, oxacillin, and monomycin. The disk diffusion test determined the antibiotic resistance of the *Bacillus* strains. The spread plate method was used for the inoculation of *Bacillus* spp. strains in L-medium. The antibiotics were then placed on agar plates. The plates were incubated in a thermostat at 37 °C for 24 hours. Zones of inhibition were recorded after cultivation. If the zone inhibition diameter was < 10 mm, the strain was considered resistant, 10–15 mm as insensitive, and 15-20 mm as sensitive. Zones greater than 25 mm indicate a high sensitivity of the microorganism to this antibiotic (Vos et al. 2009; Horváth et al. 2016).

The antagonistic activity was tested against the *Escherichia coli* strain from microorganism collection from the Engineering Center "Prombiotech" (Barnaul, Russia). This bacterium was isolated from the waste products of chickens from the poultry farm.

Agar wells method. The pour-plate method was used for the inoculation of *E. coli* culture in L-medium. The plates were allowed to solidify at room temperature. Then, 5–7 mm diameter wells were made in each plate with a drill, and 30–40 µl of bacilli strain suspension were charged into the wells. The plates were incubated in a thermostat at 37 °C for 24 hours. The radius of the inhibition zone around the charged wells was recorded after incubation.

Agar blocks method. The spread plate method was used for the inoculation of *Bacillus* spp. strains in L-medium. After that, the plates were incubated in a thermostat at 37 °C for 24 hours. Next, the agar blocks with the grown bacillus culture were cut with the help of a sterile drill and placed on the surface of the agar medium with *E. coli* culture inoculated by the pour plate method. After this, the plates were incubated in a thermostat at 37 °C for 24 hours. The radius of the inhibition zone around the agar blocks was recorded after incubation (Irkitova and Kagan 2012; Wang et al. 2017).

The studies were carried out in triplicate. The arithmetic average (x) and the standard deviation (SD) were determined for the methods of delayed antagonism and the disk diffusion test.

Results

Based on the morphology and basic properties corresponding to the genus *Bacillus*, we obtained 33 bacterial strains from 107 plant samples. Only eight isolates showed an adverse reaction to lecithinase. Strain No. 16, isolated from the rhizosphere of Cichorium, showed the highest antagonistic activity against *E. coli*. This isolate was selected for further analysis.

The colonies of *B. pumilus* 16 are of whitish to cream color (edge more transparent), matt, circular, raised, undulate, and approximately 0.5 to 0.8 cm in diameter after incubation for one day on L-agar at 37 ° C (Fig. 2).

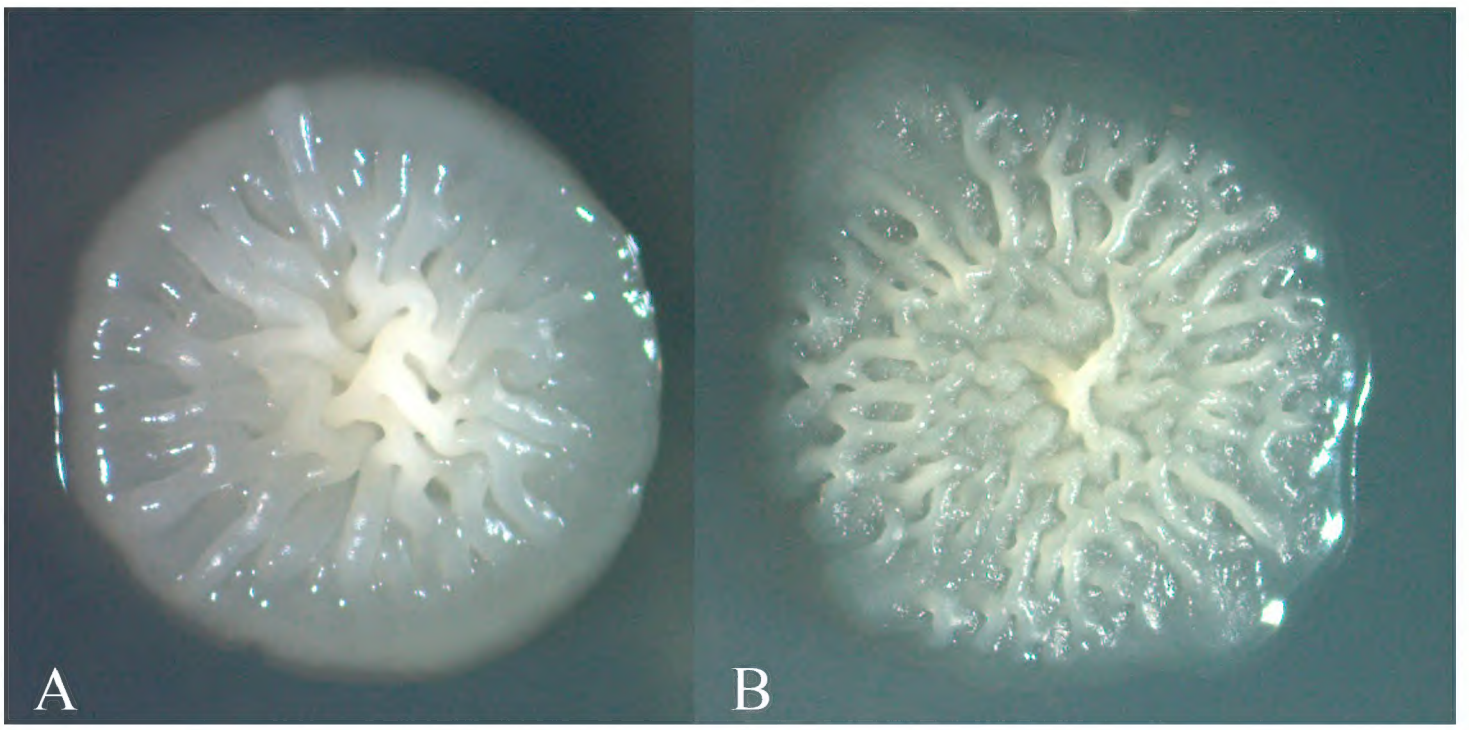

Figure 2. Morphology of the Bacillus genus colonies. **A** – the novel strain of *B. pumilus* (16). **B** – the *B. subtilis* B-1323 strain.

Like typical cultures, the novel strain, after cultivation in a shaking incubator for 18–24 hours, causes a clouding of the L-broth. A precipitate is formed in the flask after hours of rest. When *B. pumilus* 16 is cultivated in a thermostat. A folded film is formed on the surface of the broth, which is easily destroyed by shaking (Fig. 3). The optimal cultivation temperature of the new strain is 37 °C; pH is 6.8–7.0.

The microscopic study of new strains showed that these bacteria are rods with a length of about 5–7 μm and 1 μm in diameter. Cells occur singly, in pairs, and occasionally in short chains (Fig. 4, A). *B. pumilus* 16 produces oval-shaped central endospores (Fig. 4, B).

The initial selection of the nucleotide sequence (obtained by sequencing the variable regions of genes encoding 16S rRNA) using the GenBank database and the Ribosomal Database Project II (RDP-II) showed that the studied strain belongs to the following systematic groups: Bacteria, Firmicutes, Bacilli, Bacillales, Bacillaceae, Bacillus.

The results of the processing of sequences using a computer program, located on the RDB II website (Ribosomal Database Project II), designed to determine the affinity of microorganisms and build phylogenetic trees, are presented in graphical form (Fig. 5).

During the PCR reaction, the following primers were used: specific for *Bacillus pumilus* (gyrA) – Pum-f and Pum-r (Fig. 6), specific for *Bacillus safeness* (gyrA) – Saf-f and Saf-r.

A fragment of 774 bp was obtained using species-specific primers Pum-f and Pum-r. When we used primers Saf-f and Saf-r, fragments were not obtained. This made it possible to attribute the new strain to the *Bacillus pumilus*.

To study the biological activity of the new strain, we performed 29 tests (Table 1). The first five tests were taken from Bergey's Manual and allowed us to establish the belonging of the strain to the genus *Bacillus* and the level of its pathogenicity.

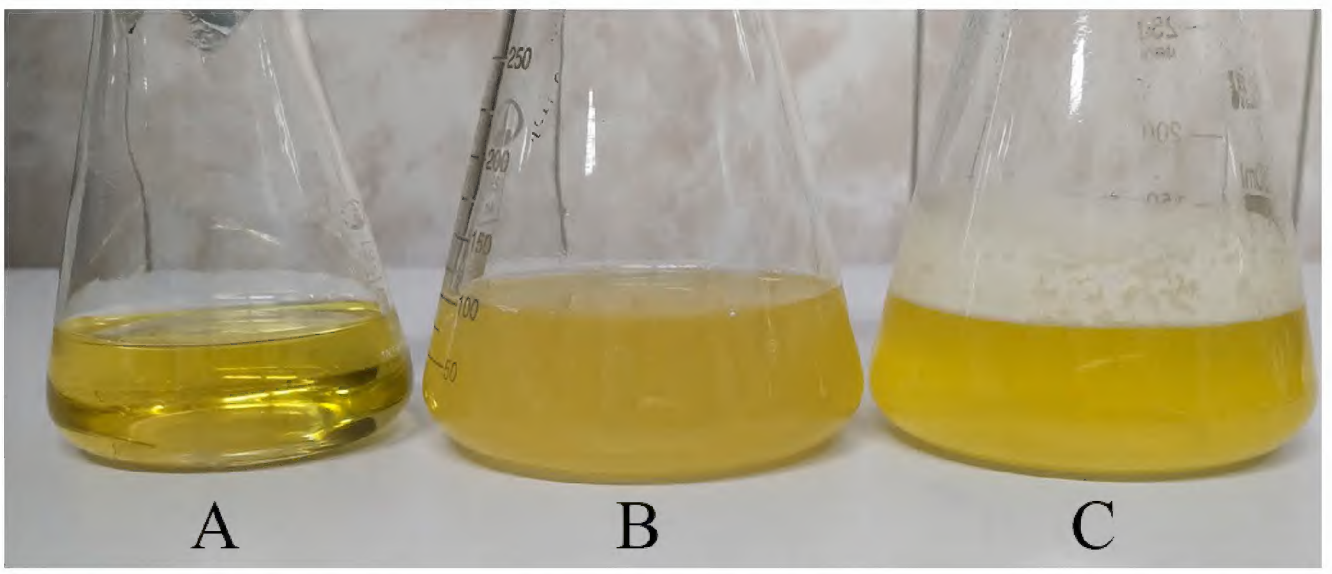

Figure 3. Growth of *B. pumilus* 16 in the L-broth. **A** – the sterile medium. **B** – 24-hour bacterial culture, which was incubated in a shaker. **C** – 24-hour bacterial culture, which was incubated in a thermostat.

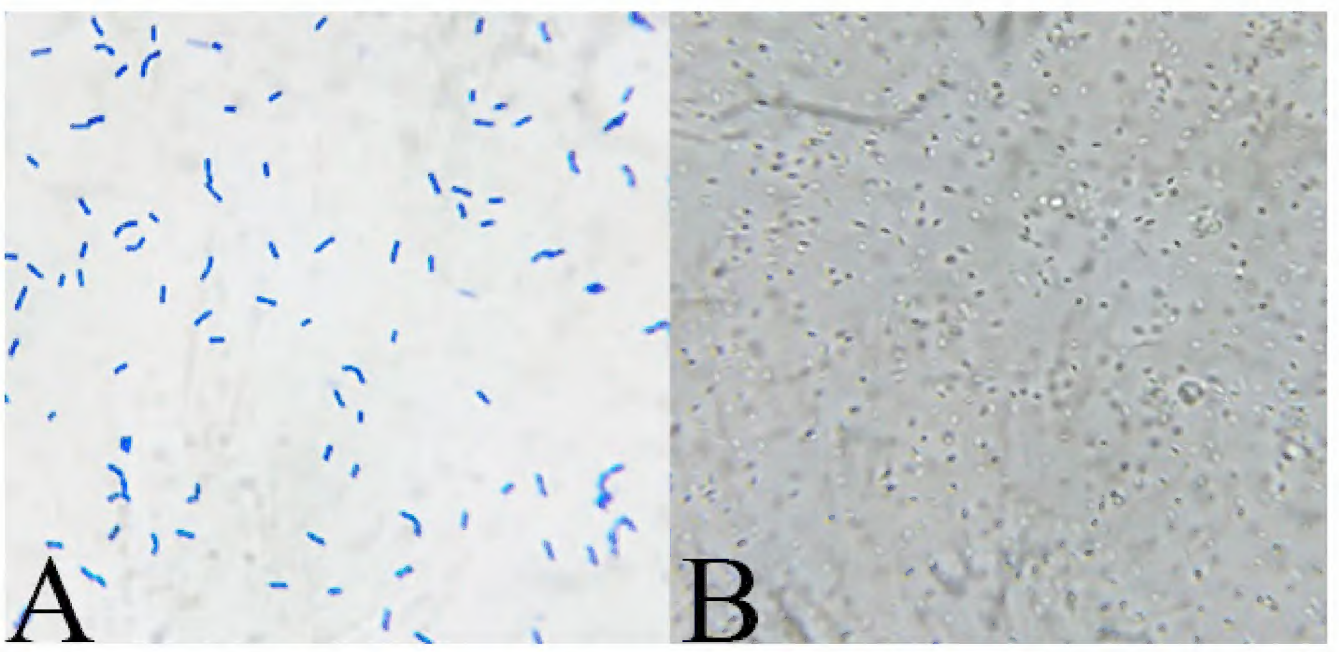

Figure 4. A novel strain under a microscope (x1000). **A** – Methylene blue-stained cells. **B** – The spores.

S000012241	0.998	0.951	1440	*Bacillus vallismortis* (T); DSM11031; AB021198
S000014133	0.998	0.951	1426	*Bacillus atrophaeus* (T); JCM9070; AB021181
S000417318	1.000	0.972	1415	*Bacillus altitudinis* (T); type strain:41KF2b; AJ831842
S000458519	1.000	0.989	1354	*Bacillus safensis* (T); FO-036b; AF234854
S000481068	1.000	0.995	1352	*Bacillus pumilus* (T); ATCC 7061; AY876289
S000734915	0.998	0.951	1389	*Bacillus amyloliquefaciens* (T); NBRC 15535; AB255669
S001014161	1.000	0.972	1443	*Bacillus stratosphericus* (T); type strain:41KF2a; AJ831841
S001014162	1.000	0.972	1443	*Bacillus aerophilus* (T); type strain:28K; AJ831844
S004007309	1.000	0.962	1426	*Bacillus xiamenensis* (T); MCCC 1A00008; JX680066

Figure 5. Sequence processing results.

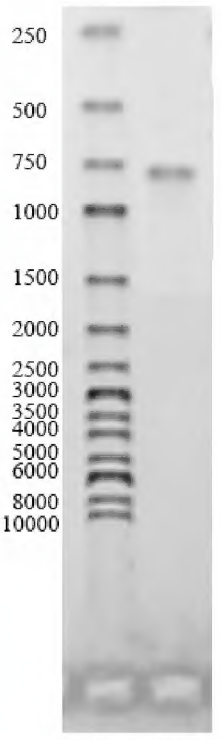

Figure 6. Results of the PCR analysis. **A** – Thermo Scientific O'GeneRuler 1 kb DNA Ladder. **B** – PCR analysis of the *B. pumilus* 16 strain using Pum-f and Pum-r primers.

Table 1. Comparison of the biochemical and physiological characteristics of *B. pumilus* 16 and type strains

Features	**Strains**				
	***B. pumilus* 16**	***B. pumilus* B-7886**	***B. pumilus* B-7917**	***B. pumilus* B-7919**	***B. subtilis* B-1323**
Gram staining	+	+	+	+	+
Spore formation	+	+	+	+	+
Anaerobic growth	–	–	–	–	–
Catalase	+	+	+	+	+
Lecithinase	–	–	–	–	–
			Substrates		
Arabinose	+	+	+	+	+
Cellobiose	+	+	+	+	+

Features	Strains				
	B. pumilus 16	*B. pumilus* B-7886	*B. pumilus* B-7917	*B. pumilus* B-7919	*B. subtilis* B-1323
Inositol	–	–	–	–	+
Mannitol	+	+	+	+	+
Mannose	+	+	+	+	+
Raffinose	–	–	–	–	+
Rhamnose	–	–	–	–	–
Salicin	+	+	+	+	+
Sorbitol	–	–	–	–	+
Sucrose	+	+	+	+	+
Trehalose	+	+	+	+	+
Xylose	–	+	+	+	–
Adonitol	–	–	–	–	–
Galactose	–	–	+	–	–
Methyl-D-Mannoside	–	–	–	–	–
Methyl-D-Glucoside	–	–	–	–	+
Inulin	–	–	–	–	+
Melezitose	–	–	–	–	–
Indole	–	–	–	–	–
ONPG	+	+	+	+	+
Nitrate	–	–	–	–	+
Arginine Dihydrolase	+	+	+	+	+
Citrate Utilization	+	–	–	–	–
Voges Proskauer	+	+	+	+	–

Notes: "+" – positive reaction, "–" – negative reaction, ONPG – O-nitrophenyl-beta-D-galactopyranoside.

Strain *B. pumilus* 16, like reference strains, is Gram positive, spore-forming, catalase positive, lecithinase negative, and not capable of growth under anaerobic conditions.

Like other *B. pumilus* strains (B-7886, B-7917, B-7919), the new rhizosphere strain can ferment such carbohydrates as arabinose, cellobiose, mannitol, mannose, salicin, sucrose, and trehalose. It also hydrolyzes ONPG and gives a positive reaction to arginine dihydrolase and the Voges-Proskauer test. However, the specificity of the new strain of *B. pumilus* 16 is the inability to ferment xylose.

Differences in biochemical characteristics between *B. pumilus* 16 and *B. subtilis* B-1323 are more significant. The studied strain does not ferment inositol, raffinose, sorbitol, methyl-D-glucoside, and inulin. It does not reduce nitrate and forms acetoin, unlike *B. subtilis* B-1323. A significant difference between new and type strains is the ability of *B. pumilus* 16 to use citrate as the sole carbon source. The novel strain was close to the type strains in terms of antibiotic resistance (Table 2).

Table 2. Antibiotic resistance (mm (x ± SD) of a novel and type strains

Antibiotics	**Strains**				
	***B. pumilus* 16**	***B. pumilus* B-7886**	***B. pumilus* B-7917**	***B. pumilus* B-7919**	***B. subtilis* B-1323**
Cephalexin	37.9±0.7 (+++)	32.7±2.5 (+++)	36.7±3.8 (+++)	35.0±1.7 (+++)	32.7±2.8 (+++)
Oleandomycin	17.1±1.9 (++)	16.7±0.6 (++)	14.7±0.6 (+)	16.3±1.2 (++)	15.7±0.6 (++)
Enrofloxacin	25.7±8.9 (+++)	27.3±0.6 (+++)	28.0±1.0 (+++)	27.0±1.0 (+++)	27.0±1.0 (+++)
Benzylpenicillin	18.5±1.2 (++)	13.3±0.6 (+)	16.3±2.1 (++)	13.7±1.2 (+)	0 (–)
Oxacillin	0 (–)	0 (–)	0 (–)	0 (–)	0 (–)
Monomycin	16.0±0.6 (++)	14.3±0.6 (+)	13.3±0.6 (+)	13.7±0.6 (+)	15.3±1.2 (++)

Note: "–" – resistance, "+" – insensitive, "++" – sensitive, "+++" – highly sensitive.

Strain *B. pumilus* 16, like all types of strains, was resistant to oxacillin. The strain *B. subtilis* B-1323 also showed resistance to benzylpenicillin. Sensitivity for other antibiotics was recorded, including high sensitivity. The least resistance to cephalexin and enrofloxacin was observed.

Table 3 shows the results of the antagonistic activity of the new strain against *E. coli*. Antagonism was fixed in both techniques for researching delayed antagonism.

Table 3. Radii of growth inhibition zones (mm (x ± SD) of *E. coli* by *B. pumilus* 16 and type strains

Methods	**Strains**				
	***B. pumilus* 16**	***B. pumilus* B-7886**	***B. pumilus* B-7917**	***B. pumilus* B-7919**	***B. subtilis* B-1323**
Agar blocks	7.3±1.5	0	0	1.1±0.1	1.5±0.5
Agar wells	5.3±0.6	0	0	2.7±0.6	0

Reference strains of *B. pumilus* B-7886 and B-7917 did not exert an inhibitory effect on *E. coli*. The antimicrobial effect of *B. subtilis* B-1323 was only recorded by the agar block method.

The strain *B. pumilus* B-7919 also showed an antagonistic effect in both methods to determine delayed antagonism, as well as *B. pumilus* 16. However, the growth blocking zones of *E. coli* by the novel strain was maximal (Fig. 7).

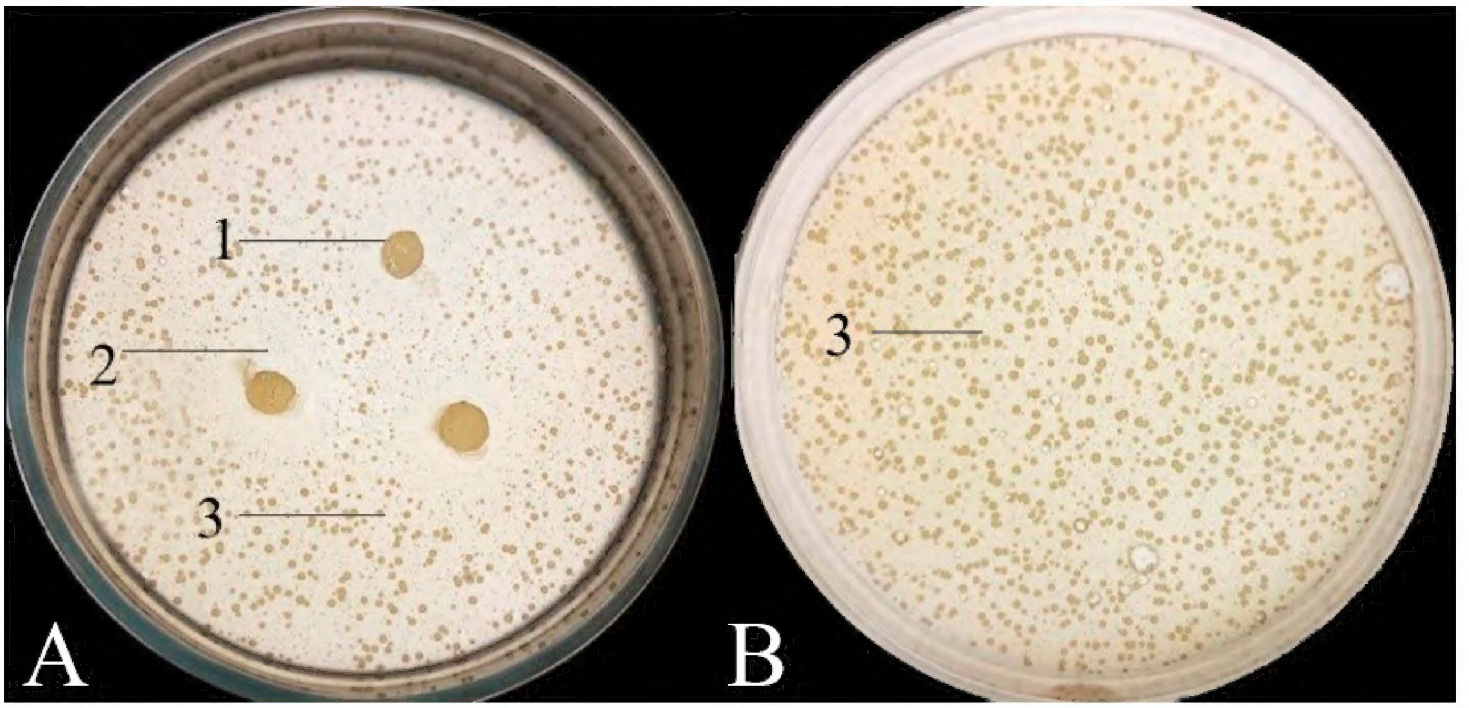

Figure 7. Antagonistic activity of *B. pumilus* 16 against *E. coli*. **A** – Plate with agar blocks. **B** – Control plate. **1**. Block with antagonist strain. **2**. The zone of growth inhibition. **3**. Test culture.

Discussion

The new strain isolated from the rhizosphere of Cichorium is marked with R-colonies typical for representatives of the *B. subtilis*, culture conditions, rod-shaped cells, and spore-forming ability. According to Bergey's Manual, the combination of physiological and biochemical features of the new strain indicates that it belongs to non-pathogenic bacilli. As a result of the sequencing of variable regions of genes encoding 16S rRNA and PCR analysis using species-specific primers (Venkataramana 2015; Mukhtar et al. 2018), the studied strain can be assigned to the *Bacillus pumilus*. Based on these results, strain *B. pumilus* 16 was deposited in VKPM under No. B-13250. We obtained the patent under No. 2, 694, 522 (RU).

The biochemical characteristics of *B. pumilus* 16 and the reference strains have significant similarities due to their belonging to the same phylogenetic group, the *Bacillus subtilis* group (BacDive, n.d.). Latorre et al. (2016) also demonstrated: "Not all *Bacillus* spp. synthesize the same enzyme types". Differences in biochemical activity are determined by the variety of ferment produced, which is associated with the variability of the bacterial genome. Furthermore, differences in the use of cer-

tain substrates as a source of carbon and energy are associated with the influence of environmental conditions to which these strains are adapted.

We have previously found (Orlova et al. 2020) that the new strain of *B. pumilus* 16 in terms of antibiotic sensitivity was close to the reference strains. According to published data, bacilli are often resistant to antibiotics of the penicillin group, including oxacillin (Andrews and Wise 2002; Owusu-Kwarteng et al. 2017), which was confirmed in our study. At the same time, new strains and reference strains of the same species were sensitive to benzylpenicillin because they did not have a penicillinase enzyme, which destroys this antibiotic. The high sensitivity of *B. pumilus* 16 and all type cultures to cephalexin can be explained by the fact that this antibiotic is resistant to β-lactamases, bacterial enzymes aimed at combating β-lactam antibiotics from the penicillin group. Enrofloxacin, related to fluoroquinolones, is effective against aerobic gram-positive bacteria, to which a new strain belongs (Ismail and Adeloju 2010; Bush and Bradford 2016; Trouchon and Lefebvre 2016).

B. pumilus 16 is sensitive to 5 of the 6 antibiotics used. Therefore, if this microorganism is used in various sectors of the national economy, it will not transfer antibiotic resistance genes to pathogenic bacteria. On the other hand, this fact makes it impossible to use a probiotic containing the *B. pumilus* 16 strain during antibiotic therapy of the antibiotic compounds studied (Wintersdorff et al. 2016; Kerna and Brown 2018; Sun et al. 2019).

The new strain is marked with higher antagonistic activity compared to the reference strains. This may be because the strain studied is natural and, therefore, more stable, since the struggle for existence is constant under conditions of high density of microorganisms on the root surface. This leads to the development of various mechanisms of suppression of competitors (Krober et al. 2014; Andrić et al. 2020). Furthermore, the *B. pumilus* 16 strain, unlike type strains, was recently isolated. Thus, it has not yet lost its effectiveness under laboratory conditions.

Liu et al. (2019) confirmed that surfactin synthesized by *B. subtilis* is effective against *E. coli*. Xiu et al. (2017) isolated from *B. pumilus* a surfactin-like antibiotic, pumilacidin, which was effective against *Vibrio alginolyticus*. Saggese et al. (2018) proved that pumylacidin is active against *Staphylococcus aureus*. This indicates that bacilli synthesize various substances with antimicrobial activity with a wide spectrum of antimicrobial activity. According to our previous results (Funk et al. 2019), *B. pumilus* 16 inhibited the growth of *E. coli* in both techniques to investigate delayed antagonism. Successful inhibition of *E. coli* growth by *B. pumilus* 16 using various methods to determine antagonistic activity may indicate the strain's ability to synthesize different antimicrobial compounds. Because various mechanisms of antagonism are triggered. But more research is needed to confirm this assumption.

Conclusion

Thus, a new strain 16, which was isolated from the rhizosphere of Cichorium, was identified as *B. pumilus*. The strain was deposited in VKPM (Russian National Collection of Industrial Microorganisms) under No. B-13250 and patented under No. 2, 694, 522 (RU). *B. pumilus* 16 did not show resistance to antibiotics of the aminoglycoside group, cephalosporins, and benzylpenicillin (the zone of growth inhibition ranged from 16.0 to 37.9 mm). The strain studied in terms of antagonistic activity against *E. coli* is superior to the reference strains *B. pumilus* B-7886, *B. pumilus* B-7917, *B. pumilus* B-7919, and *B. subtilis* B-1323. In connection with the above facts, the novel strain of *B. pumilus* 16 is a valuable biological resource. It is promising for use as an active component in biological preparations.

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